

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant(s) : Jean-Christophe Audonnet, *et al*  
U.S. Appln. No. : 09/943,443  
U.S. Filing Date : 08-30-2001  
Title of Invention : Feline polynucleotide vaccine formula  
Confirm No. : 9956  
Examiner : CHEN, STACY BROWN  
Art Unit : 1648

745 Fifth Avenue, New York, New York 10151

**DECLARATION CERTIFYING ACCURACY OF  
ENGLISH TRANSLATION OF PRIORITY DOCUMENT**

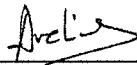
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Dear Sir:

I, Béatrice M. Aveline of Cabinet Plasseraud, declare and state that:

1. I am conversant with the English and French languages and am a competent translator thereof.
2. I certify that to the best of my knowledge and belief the following is a true and correct translation of the document in French language filed for a French Patent application under Application No. 96 09337 on July 19, 1996.
3. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true. These statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: Oct. 21<sup>st</sup>, 2008



Béatrice M. Aveline, Ph.D.

# TRANSLATION

FRENCH REPUBLIC  
NATIONAL INSTITUTE  
OF INDUSTRIAL PROPERTY

PARIS

Publication No.: 2 751 223

(to be used only  
for publication orders)

National Registration No.: 96 09337

Int Cl<sup>7</sup>: A 61 K 48/00, A 61 K 3923, 39/205 // C 12 N 15/85C

## PATENT APPLICATION

A1

Filing Date: 19.07.96

Applicant(s): RHONE MERIEUX SOCIETE  
ANONYME - FR

Priority:

Inventor(s): AUDONNET JEAN-CHRISTOPHE  
FRANCIS, BOUCHARDON ANNABELLE, BAUDU  
PHILIPPE et RIVIERE

Date of availability of the application  
to the public: 23.01.98 Bulletin 98/04

List of documents cited in the preliminary  
search report: see at the end of the present  
document

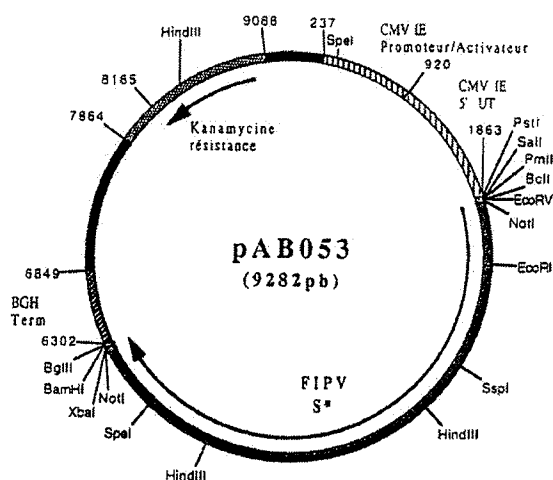
References cited in other corresponding  
national applications:

Assignee(s):

Agent(s): CABINET LAVOIX

## FELINE POLYNUCLEOTIDE VACCINE FORMULA

A cat vaccine formula including at least three polynucleotide vaccine valencies that each include a plasmid containing a cat pathogen valency gene capable of being expressed in vivo in host cells. Said valencies are selected from the group which consists of feline leukaemia virus, panleukopenia virus, infectious peritonitis virus, coryza virus, calicivirus disease virus, feline immunodeficiency virus and optionally rabies virus. Said plasmids include one or more genes per valency, and said genes are selected from the group which consists of env and gag for feline leukaemia, VP2 for panleukopenia, modified S, M and N for infectious peritonitis, gB and gD for coryza, capsid for calicivirus disease, env and gag/pro for feline immunodeficiency and G for rabies.



## FELINE POLYNUCLEOTIDE VACCINE FORMULA

The present invention relates to a vaccine formula allowing the vaccination of cats against a number of pathologies. It also relates to a corresponding method of vaccination.

Associations of vaccines against certain canine viruses have already been proposed in the past.

The associations developed so far were prepared from inactivated vaccines or live vaccines and, optionally, from mixtures of such vaccines. Their development raises problems of compatibility between valencies and of stability. It is indeed necessary to ensure both the compatibility between the different vaccine valencies, whether from the point of view of the different antigens used or from the point of view of the formulations themselves, especially in the case where both inactivated vaccines and live vaccines are combined. The problem of the conservation of such combined vaccines and of their safety especially in the presence of an adjuvant also exists. These vaccines are in general quite expensive.

Patent Applications WO-A-90 11092, WO-A-93 19183, WO-A-94 21797 and WO-A-95 20660 have made use of the recently developed technique of polynucleotide vaccines. It is known that these vaccines use a plasmid capable of expressing, in the host cells, the antigen inserted into the plasmid. All routes of administration have been proposed (intraperitoneal, intravenous, intramuscular, transcutaneous, intradermal, mucosal and the like). Various vaccination means can also be used, such as DNA deposited at the surface of gold particles and projected so as to penetrate into the animals' skin (Tang et al., Nature 356, 152-154, 1992) and liquid jet injectors which make it possible to transfect at the same time the skin, the muscle, the fatty tissues and the mammary tissues (Furth et al., Analytical Biochemistry, 205, 365-368, 1992).

Polynucleotide vaccines may also use naked DNAs as well as DNAs formulated, for example, inside cationic lipids or liposomes.

The invention therefore proposes to provide a multivalent vaccine formula that makes possible to ensure vaccination against a number of feline pathogenic viruses.

Another objective of the invention is to provide such a vaccine formula combining different valencies while exhibiting all the criteria required for mutual compatibility and stability of the valencies.

Another objective of the invention is to provide such a vaccine formula which makes it possible to combine different valencies in the same vehicle.

Another objective of the invention is to provide such a vaccine which is easy and inexpensive to use.

Yet another objective of the invention is to provide a method for vaccinating cats allowing protection, including multivalent protection, with a high level of efficiency and of long duration, as well as good safety.

The subject of the present invention is therefore a vaccine formula intended for cats, comprising at least three polynucleotide vaccine valencies each comprising an integrating plasmid, so as to express it in vivo in the host cells, a gene of a feline pathogen valency, these valencies being selected from the group consisting of feline leukaemia virus (FeLV), panleukopenia virus (FPV), infectious peritonitis virus (FIPV), coryza virus (FHV), caliciviro-sis virus (FCV), feline immunodeficiency virus (FIV) and possibly rabies virus (rhabdovirus), the plasmids comprising, for each valency, one or more of the genes selected from the group consisting of env and gag/pol for the feline leukaemia, VP2 for the panleukopenia, modified S (or S\*) and M for the infectious peritonitis, gB and gD for the coryza, capsid for the caliciviro-sis, env and gag/pro for the feline immunodeficiency and G for the rabies.

Valency in the present invention is understood to mean at least one antigen providing protection against the virus of the pathogen considered, wherein the valency may contain, as subvalency, one or more modified or natural genes from one or more strains of the pathogen considered.

Gene of a pathogenic agent is understood to mean not only the whole gene but also the various nucleotide sequences, including fragments which retain the ability to induce a protective response. The term gene encompasses nucleotide sequences equivalent to those precisely described in the examples, i.e. the sequences which are different but which encode the same protein. It also encompasses the nucleotide sequences of other strains of the pathogen

considered, providing a cross-protection or a protection specific for a strain or for a strain group. It also encompasses the nucleotide sequences which have been modified in order to facilitate the in vivo expression by the host animal but which encode the same protein.

Preferably, the vaccine formula according to the invention comprises the panleukopenia, coryza and calicivirolosis valencies.

It will be possible to add the feline leukaemia, feline immunodeficiency and/or infectious peritonitis valencies.

As far as the coryza valency is concerned, it is preferable to use the two genes coding for gB and gD integrated in different plasmids or in a single plasmid, or to use either of these genes.

For the feline leukaemia valency, use is preferably made of the two env and gag/pol genes integrated into two different plasmids or into a single plasmid, or the env gene alone.

For the feline immunodeficiency valency, use will preferably be made of the two env and gag/pro genes in different plasmids or in a single plasmid, or only one of these genes. Still more preferably, the FeLV-A env gene and the FeLV-A and FeLV-B env genes are used.

For the infectious peritonitis valency, use is preferably made of the two M and modified S genes together in two different plasmids or in a single plasmid, or either of these genes. S will be modified in order to inactivate the major facilitating epitopes, preferably according to the teaching of Patent PCT/FR95/01128.

The vaccine formula according to the invention can be presented in a dose volume of between 0.1 and 3 ml and in particular between 0.5 and 1 ml.

The dose will be generally between 10 ng and 1 mg, preferably between 100 ng and 500 µg and still more preferably between 1 µg and 250 µg per plasmid type.

Use will preferably be made of naked plasmids simply placed in the vaccination vehicle which will be, in general, physiological saline (0.9% NaCl), ultrapure water, TE buffer and the like. All the polynucleotide vaccine forms described in the prior art can of course be used.

Each plasmid comprises a promoter capable of ensuring the expression into the host cells of the gene inserted, under its control. This will be in general a strong eukaryotic promoter

and in particular a cytomegalovirus early CMV-IE promoter of human or murine origin, or optionally of another origin such as rats, pigs and guinea pigs.

More generally, the promoter may be either of viral origin or of cellular origin. As viral promoter, there may be mentioned the SV40 virus early or late promoter or the Rous sarcoma virus LTR promoter. It may also be a promoter from the virus from which the gene is derived, for example the gene's own promoter.

As cellular promoter, there may be mentioned the promoter of a cytoskeleton gene, such as for example the desmin promoter (Bolmont et al., *Journal of Submicroscopic Cytology and Pathology*, 1990, 22, 117-122; and Zhenlin et al., *Gene*, 1989, 78, 243-254), or alternatively the actin promoter.

When several genes are present in the same plasmid, these may be presented in the same transcription unit or in two different units.

The combination of the different vaccine valencies according to the invention may preferably be achieved by mixing the polynucleotide plasmids expressing the antigen(s) of each valency, but it is also possible to have the same plasmid express antigens of several valencies.

The subject of the invention is also monovalent vaccine formulae comprising one or more plasmids encoding one or more genes from one of the viruses cited above, the genes being those described above. Besides their monovalent character, these formulae may possess the characteristics stated above regarding the choice of the genes, their combinations, the composition of the plasmids, the dose volumes, the doses and the like.

The monovalent vaccine formulae may also be used (i) for the preparation of a polyvalent vaccine formula as described above, (ii) individually against the actual pathology, (iii) combined with a vaccine of another type (live or inactivated whole, recombinant, subunit) against another pathology, or (iv) as booster for a vaccine as described below.

The subject of the present invention is in fact also the use of one or more plasmids according to the invention for the manufacture of a vaccine intended to vaccinate cats primovaccinated by means of a first conventional vaccine (monovalent or multivalent) as known in the prior art, in particular, selected from the group consisting of a live whole vaccine, an inactivated whole vaccine, a subunit vaccine, a recombinant vaccine, this first vaccine having (i.e.,

containing or capable of expressing) the antigen(s) encoded by the plasmid(s) or antigen(s) providing cross-protection.

Remarkably, the polynucleotide vaccine has a potent booster effect which results in an amplification of the immune response and the acquisition of a long-lasting immunity.

In general, the primo-vaccination vaccines can be selected from commercial vaccines available from various veterinary vaccine producers.

The subject of the invention is also a vaccination kit assembling together a primo-vaccination vaccine as described above and a vaccine formula according to the invention for the booster. It also relates to a vaccine formula according to the invention accompanied by a notice indicating the use of this formula as a booster for a primo-vaccination as described above.

The subject of the present invention is also a method for vaccinating cats, comprising the administration of an effective vaccine formula as described above. This vaccination method comprises the administration of one or more doses of the vaccine formula, wherein these doses can be administered successively over a short period of time and/or successively at widely spaced intervals.

The vaccine formulae according to the invention can be administered in the context of this method of vaccination, by different routes of administration proposed in the prior art for polynucleotide vaccination and by means of known techniques of administration.

The subject of the invention is also a method of vaccination consisting in performing a primo-vaccination as described above and a booster with a vaccine formula according to the invention.

In a preferred embodiment of the process according to the invention, in a first instance, an animal is administered an effective dose of the vaccine of the conventional, especially inactivated, live, attenuated or recombinant, type, or alternatively a subunit vaccine, so as to provide a primo-vaccination, and, after a period preferably of 2 to 6 weeks, is administered the polyvalent or monovalent vaccine according to the invention.

The efficiency of presentation of the antigens to the immune system varies according to the tissues. In particular, the mucous membranes of the respiratory track serve as barriers to the entry of pathogens and are associated with lymphoid tissues which support local immunity.

Administration of a vaccine by contact with the mucous membranes, in particular the buccal mucous membrane, the pharyngeal mucous membrane and the mucous membrane of the bronchial region, is of great interest for the vaccination against respiratory and digestive pathologies.

Consequently, administration using the mucosal routes is a preferred mode of administration for the invention, using in particular nebulization or spray or drinking water. It will be possible to apply the vaccine formulae and the vaccination methods according to the invention in this context.

The invention will now be described in greater detail with the aid of the embodiments of the invention taken with reference to the accompanying drawings.

**List of figures**

Figure No. 1:	Plasmid pVR1012
Figure No. 2:	Plasmid pPB179
Figure No. 3:	Sequence of the FOLV-B env gene
Figure No. 4:	Plasmid pPB180
Figure No. 5:	Sequence of the FeLV-A virus gag/pol gene (Glasgow-1 strain)
Figure No. 6:	Plasmid pPB181
Figure No. 7:	Plasmid pAB009
Figure No. 8:	Plasmid pAB053
Figure No. 9:	Plasmid pAB052
Figure No. 10:	Plasmid pAB056
Figure No. 11:	Plasmid pAB028
Figure No. 12:	Plasmid pAB029
Figure No. 13:	Plasmid pAB010
Figure No. 14:	Plasmid pAB030
Figure No. 15:	Plasmid pAB083
Figure No. 16:	Plasmid pAB041

**Sequence listing SEQ ID No.**

SEQ ID No. 1:	Oligonucleotide PB247
---------------	-----------------------



SEQ ID No. 2	Oligonucleotide PB249
SEQ ID No. 3:	Oligonucleotide PB281
SEQ ID No. 4:	Oligonucleotide PB282
SEQ ID No. 5:	Sequence of the FELV-B virus env gene
SEQ ID No. 6:	Oligonucleotide PB283
SEQ ID No. 7:	Oligonuclootide PB284
SEQ ID No. 8:	Sequence of the FeLV-A virus gag/pol gene (Glasgow-1 strain)
SEQ ID No. 9:	Oligonucleotide AB021
SEQ ID No. 10:	Oligonucleotide AB024
SEQ ID No. 11:	Oligonucleotide AB103
SEQ ID No. 12:	Oligonucleotide AB112
SEQ ID No. 13:	Oligonucleotide AB113
SEQ ID No. 14:	Oligonucleotide AB104
SEQ ID No. 15:	Oligonucleotide AB101
SEQ ID No. 16:	Oligonucleotide AB102
SEQ ID No. 17:	Oligonuclootide AB106
SEQ ID No. 18:	Oligonucleotide AB107
SEQ ID No. 19:	Oligonucleotide AB061
SEQ ID No. 20:	Oligonucleotide AB064
SEQ ID No. 21:	Oligonucleotide AB065
SEQ ID No. 22:	Oligonuclootide AB066
SEQ ID No. 23:	Oligonucleotide AB025
SEQ ID No. 24:	Oligonucleotide AB026
SEQ ID No. 25:	Oligonucleotide AB067
SEQ ID No. 26:	Oligonucleotide AB070
SEQ ID No. 27:	Oligonucleotide AB154
SEQ ID No. 28:	Oligonucleotide AB155
SEQ ID No. 29:	Oligonucleotide AB011
SEQ ID No. 30:	Oligonucleotide AB012

## **EXAMPLES**

### **Example 1: Culture of the Viruses**

The viruses are cultured on the appropriate cellular system until a cytopathic effect is obtained. The cellular systems to be used for each virus are well known to persons skilled in the art. Briefly, the cells sensitive to the virus used, cultured in Eagle's minimum essential medium (MEM medium) or another appropriate medium, are inoculated with the viral strain studied using a multiplicity of infection of 1. The infected cells are then incubated at 37°C. for a time necessary for the appearance of a complete cytopathic effect (on average 36 hours).

### **Example 2: Extraction of the Viral Genomic DNAs**

After culture, the supernatant and the lysed cells are harvested and the entire viral suspension is centrifuged at 1000 g for 10 minutes at +4°C so as to remove the cellular debris. The viral particles are then harvested by ultracentrifugation at 400,000 g for 1 hour at +4°C. The pellet is taken up in a minimum volume of buffer (10 mM Tris, 1 mM EDTA). This concentrated viral suspension is treated with proteinase K (100 µg/ml final) in the presence of sodium dodecyl sulphate (SDS) (0.5% final) for 2 hours at 37°C. The viral DNA is then extracted with a mixture of phenol/chloroform and then precipitated with 2 volumes of absolute ethanol. After leaving the sample overnight at -20°C, the DNA is centrifuged at 10,000 g for 15 minutes at +4°C. The DNA pellet is dried and then taken up in a minimum volume of sterile ultrapure water. It can then be digested with restriction enzymes.

### **Example 3: Isolation of the Viral Genomic RNAs**

The RNA viruses were purified according to techniques well known to one skilled in the art. The genomic viral RNA of each virus was then isolated using the "guanidium thiocyanate/phenol-chloroform" extraction technique described by P. Chomczynski and N. Sacchi (Anal. Biochem., 1987, 162, 156-159).

### **Example 4: Molecular Biology Techniques**

All the constructions of plasmids were carried out using standard molecular biology techniques described by J. Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold

Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989). All the restriction fragments used for the present invention were isolated using the "GeneClean" kit (BIO 101 Inc. La Jolla, Calif.).

#### **Example 5: RT-PCR Technique**

Specific oligonucleotides (comprising restriction sites at their 5' ends to facilitate the cloning of the amplified fragments) were synthesized such that they completely cover the coding regions of the genes to be amplified (see specific examples). The reverse transcription (RT) reaction and polymerase chain reaction (PCR) were carried out according to standard techniques (Sambrook J. et al., 1989). Each RT-PCR reaction was performed with a pair of specific amplimers and using, as template, the extracted viral genomic RNA. The complementary DNA amplified was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) before being digested with restriction enzymes.

#### **Example 6: Plasmid pVR1012**

The plasmid pVR1012 (Figure No. 1) was obtained from Vical Inc., San Diego, Calif., USA. Its construction has been described in J. Hartikka et al. (Human Gene Therapy, 1996, 7, 1205-1217).

#### **Example 7: Construction of the Plasmid pPB179 (FeLV-A Virus Env Gene)**

An RT-PCR reaction according to the technique described in Example 5 was carried out with feline leukaemia virus (FeLV-A) (Glasgow-1 strain) genomic RNA (M. Stewart et al. J. Virol. 1986. 58. 825-834), prepared according to the technique described in Example 3, and with the following oligonucleotides:

PB247 (29 mer) (SEQ ID No. 1)

5'TTTGTCGACCATGGAAAGTCCAACGCACC3'

PB249 (28 mer) (SEQ ID No. 2)

5'TTTGGATCCTCATGGTCGGTCCGGATCG3'

so as to amplify a 1947 bp fragment containing the gene encoding the Env glycoprotein from the FeLV-A virus (Glasgow-1 strain) in the form of a Sall-BamHI fragment. After purification, the RT-PCR product was digested with Sall and BamHI in order to give a 1935 bp Sall-BamHI fragment.

This fragment was ligated with the vector pVR1012 (Example 6), previously digested with Sall and BamHI, to give the plasmid pPB179 (6804 bp) (Figure No. 2).

**Example 8: Construction of the Plasmid pPB180 (FeLV-S Virus Env Gene)**

An RT-PCR reaction according to the technique described in Example 5 was carried out with feline leukaemia virus (FeLV-B subtype) genomic RNA, prepared according to the technique described in Example 3, and with the following oligonucleotides:

PB281 (29 mer) (SEQ ID No. 3)

5'TTTGTCGACATGGAAGGTCCAACGCACCC3'

PB282 (32 mer) (SEQ ID No. 4)

5'TTGGATCCTCATGGTCGGTCCGGATCATATTG3'

so as to amplify a 2005 bp fragment containing the gene encoding the Env glycoprotein from the FeLV-B virus (Figure No. 3 and SEQ ID No. 5) in the form of a Sall-BamHI fragment. After purification, the RT-PCR product was digested with Sall and BamHI in order to give a 1995 bp Sall-BamHI fragment.

This fragment was ligated with the vector pVR1012 (Example 6), previously digested with Sall and BamHI, to give the plasmid pPB180 (6863 bp) (Figure No. 4).

**Example 9: Construction of the Plasmid pPB181 (FeLV gag/pol gene)**

An RT-PCR reaction according to the technique described in Example 5 was carried out with the feline leukaemia virus (FeLV-A subtype) (Glasgow-1 strain) genomic RNA, prepared according to the technique described in Example 3, and with the following oligonucleotides:

PB283 (33 mer) (SEQ ID No. 6)

5'TTGTCGACATGTCTGGAGCCTCTAGTGGGACAG3'

PB284 (42 mer) (SEQ ID No. 7)

5'TTGGATCCTTATTTAATTACTGCAGTTCCAAGGAACTCTC3'

so as to amplify a 3049 bp fragment containing the sequence encoding the Gag protein and the 5' part of the sequence encoding the Pol protein from the FeLV-A virus (Glasgow-1 strain) (Figure

No. 5 and SEQ ID No. 8) in the form of a SalI-BamHI fragment. After purification, the RT-PCR product was digested with SalI and BamHI to give a 3039 bp SalI-BamHI fragment.

This fragment was ligated with the vector pVR1012 (Example 6), previously digested with SalI and BamHI, to give the plasmid pPB181 (7908 bp) (Figure No. 6).

#### **Example 10: Construction of the Plasmid pAB009 (FPV VP2 gene)**

A PCR reaction was carried out with the feline panleukopaenia virus (193 strain) genomic DNA (J. Martyn et al., J. Gen. Virol. 1990, 71. 2747-2753), prepared according to the technique of Example 2, and with the following oligonucleotides:

AB021 (34 mer) (SEQ ID No. 9)

5'TGCTCTAGAGCAATGAGTGATGGAAGCAGTTCAAC3'

AB024 (33 mer) (SEQ ID No. 10)

5'CGCGGATCCATTAATATAATTTTCTAGGTGCTA3'

so as to amplify a 1776 bp fragment containing the gene encoding the FPV VP2 capsid protein. After purification, the PCR product was digested with XbaI and BamHI in order to give a 1764 bp XbaI-BamHI fragment.

This fragment was ligated with the vector pVR1012 (Example 6), previously digested with XbaI and BamHI, to give the plasmid pAB009 (6664 bp) (Figure No. 7).

#### **Example 11: Construction of the Plasmid pAB053 (FIPV S\* gene)**

An RT-PCR reaction according to the technique described in Example 5 was carried out with the feline infectious peritonitis (FIP) virus (79-1146 strain) genomic RNA (R. de Groot et al., J. Gen. Virol. 1987. 68. 2639-2646), prepared according to the technique described in Example 3, and with the following oligonucleotides:

AB103 (38 mer) (SEQ ID No. 11)

5'ATAAGAATGCGGCCGCATGATTGTGCTCGTAACTTGCC3'

AB112 (25 mer) (SEQ ID No. 12)

5'CGTACATGTGGAATTCCACTGGTTG3'

so as to amplify the sequence of the 5' part of the gene encoding the virus S glycoprotein in the form of an NotI-EcoRI fragment. After purification, the 492 bp RT-PCR product was digested with NotI and EcoRI in order to liberate a 467 bp NotI-EcoRI fragment (fragment A).

The plasmid pJCA089 (Patent Application PCT/FR95/01128) was digested with EcoRI and SpeI in order to liberate a 3378 bp fragment containing the central part of the gene encoding the FIP virus modified S glycoprotein (fragment B).

An RT-PCR reaction according to the technique described in Example 5 was carried out with the FIP virus (79-1146 strain) genomic RNA, prepared according to the technique described in Example 3, and with the following oligonucleotides:

AB113 (25 mer) (SEQ ID No. 13)

5'AGAGTTGCAACTAGTTCTGATTTTG3'

AB104 (37 mer) (SEQ ID No. 14)

5'ATAAGAATGCGGCCGCTTAGTGGACATGCACTTTTTC3'

so as to amplify the sequence of the 3' part of the gene encoding the FIP virus S glycoprotein in the form of an SpeI-NotI fragment. After purification, the 543 bp RT-PCR product was digested with SpeI and NotI in order to liberate a 519 bp SpeI-NotI fragment (fragment C).

Fragments A, B and C were then ligated together into the vector pVR1012 (Example 6), previously digested with NotI, to give the plasmid pAB053 (9282 bp), which contains the modified S gene in the correct orientation relative to the promoter (Figure No. 8).

#### **Example 12: Construction of the Plasmid pAB052 (FIPV M gene)**

An RT-PCR reaction according to the technique described in Example 5 was carried out with the feline infectious peritonitis (FIP) virus (79-1146 strain) genomic RNA (H. Vennema et al., Virology. 1991, 181. 327-335), prepared according to the technique described in Example 3, and with the following oligonucleotides:

AB101 (37 mer) (SEQ ID No. 15)

5'ACGCGTCGACCCACCATGAAGTACATTTTGCTAATAC3'

AB102 (36 mer) (SEQ ID No. 16)

5'CGCGGATCCTTACACCATATGTAATAATTTTTCATG3'

so as to precisely isolate the gene encoding the FIP virus M glycoprotein in the form of a Sall-BamHI fragment. After purification, the 812 bp RT-PCR product was digested with Sall and BamHI in order to liberate a 799 bp Sall-BamHI fragment. This fragment was then ligated into the vector pVR1012 (Example 6), previously digested with Sall and BamHI, to give the plasmid pAB052 (5668 bp) (Figure No. 9).

#### **Example 13: Construction of the Plasmid pAB056 (FIPV N gene)**

An RT-PCR reaction according to the technique described in Example 5 was carried out with the feline infectious peritonitis (FIP) virus (79-1146 strain) genomic RNA (H. Vennema et al., Virology. 1991, 181. 327-335), prepared according to the technique described in Example 3, and with the following oligonucleotides:

AB106 (35 mer) SEQ ID No. 17)

5'ACGCGTCGACGCCCTGGCCACACAGGGACAACGCG3'

AB107 (36 mer) (SEQ ID No. 18)

5'CGCGGATCCTTAGTCGTAACCTCATCAATCATCTC3'

so as to precisely isolate the gene encoding the FIP virus N protein in the form of a Sall-BamHI fragment. After purification, the 1156 bp RT-PCR product was digested with Sall and BamHI in order to liberate a 1143 bp Sall-BamHI fragment. This fragment was then ligated into the vector pVR1012 (Example 6), previously digested with Sall and BamHI, to give the plasmid pAB056 (6011 bp) (Figure No. 10).

#### **Example 14: Construction of the Plasma pAB028 (FEV gB gene)**

A PCR reaction was carried out with the feline herpesvirus (FHV-1) (C27 strain) genomic DNA (S. Spatz et al. Virology. 1993. 197. 125-36) prepared according to the technique of Example 2, and with the following oligonucleotides:

AB061 (36 mer) (SEQ ID No. 19)

5'AAAACTGCAGAATCATGTCCACTCGTGGCGATCTTG3'

AB064 (40 mer) (SEQ ID No. 20)

5'ATAAGAATGCGGCCCCCTTAGACAAGATTTGTTTCAGTATC3'

so as to amplify a 2856 bp fragment containing the gene encoding the FHV-1 virus gB glycoprotein in the form of a PstI-NotI fragment. After purification, the PCR product was digested with PstI and NotI to give a 2823 bp PstI-NotI fragment.

This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and NotI, to give the plasmid pAB028 (7720 bp) (Figure No. 11).

#### **Example 15: Construction of the Plasmid pAB029 (FHV gD gene)**

A PCR reaction was carried out with the feline herpesvirus (FHV-1) (C-27 strain) genomic DNA (S. Spatz et al. J. Gen. Virol. 1994. 75. 1235-1244), prepared according to the technique described in Example 2 and with the following oligonucleotides:

AB065 (36 mer) (SEQ ID No. 21)

5'AAAACCTGCAGCCATGATGACACGTCTACATTTTGTG3'

AB066 (33 mer) (SEQ ID No. 22)

5'GGAAGATCTTTAAGGATGGTGAGTTGTATGTAT3'

so as to amplify the gene encoding the FHV-1 virus gD glycoprotein in the form of a PstI-BglII fragment. After purification, the 1147 bp PCR product was digested with PstI and BglII in order to isolate a 1129 bp PstI-BglII fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and BglII, to give the plasmid pAB029 (5982 bp) (Figure No. 12).

#### **Example 16: Construction of the Plasmid pAB010 (FCV C gene)**

An RT-PCR reaction according to the technique described in Example 5 was carried out with the feline calicivirus (FCV) (F9 strain) genomic RNA (M. Carter et al. Virology. 1992. 190. 443-448), prepared according to the technique described in Example 3, and with the following oligonucleotides:

AB025 (33 mer) (SEQ ID No. 23)

5'ACGCGTCGACGCATCTGCTCAACCTGCGCTAAC3'

AB026 (31 mer) (SEQ ID No. 24)

5'CGCGGATCCTCATAACTTAGTCATGGGACTC3'



so as to isolate the gene encoding the FCV virus capsid protein in the form of a Sall-BamHI fragment. After purification, the 2042 bp RT-PCR product was digested with Sall and BamHI in order to isolate a 2029 bp Sall-BamHI fragment. This fragment was ligated with the vector PVR1012 (Example 6), previously digested with Sall and BamHI, to give the plasmid pAB010 (6892 bp) (Figure No. 13).

#### **Example 17: Construction of the Plasmid pAB030 (FIV env gene)**

An RT-PCR reaction according to the technique of Example 5 was carried out with the feline immunodeficiency virus (FIV) (Petaluma strain) genomic RNA (R. Olmsted, et al. Proc. Natl. Acad. Sci. USA. 1989. 86. 8083-8096), prepared according to the technique of Example 3, and with the following oligonucleotides:

AB067 (36 mer) (SEQ ID No. 25)

5'AAAACTGCAGAAGGAATGGCAGAAGGATTTGCAGCC3'

AB070 (36 mer) (SEQ ID No. 26)

5'CGCGGATCCTCATTCCTCCTCTTTTTCAGACATGCC3'

so as to amplify a 2592 bp fragment containing the gene encoding the Env glycoprotein from the FIV virus (Petaluma strain) in the form of a PstI-BamHI fragment. After purification, the RT-PCR product was digested with PstI and BamHI to give a 2575 bp PstI-BamHI fragment.

This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and BamHI, to give the plasmid pAB030 (7436 bp) (Figure No. 14).

#### **Example 18: Construction of the Plasmid pAB083 (FIV gag/pro gene)**

An RT-PCR reaction according to the technique described in Example 5 was carried out with the feline immunodeficiency virus (FIV) (Petaluma strain) genomic RNA (R. Olmsted et al. Proc. Natl. Acad. Sci. USA. 1989. 86. 8088-8096), prepared according to the technique described in Example 3, and with the following oligonucleotides:

AB154 (32 mer) (SEQ ID No. 27)

5'ACGCGTCGACATGGGGAATGGACAGGGGCGAG3'

AB155 (33 mer) (SEQ ID No. 28)

5'TGAAGATCTTCACTCATCCCCTTCAGGAAGAGC3'

so as to amplify a 4635 bp fragment containing the gene encoding the Gag and Pro proteins from the FIV virus (Petaluma strain) in the form of a Sall-BglII fragment. After purification, the RT-PCR product was digested with Sall and BglII to give a 4622 bp Sall-BglII fragment.

This fragment was ligated with the vector pVR1012 (Example 6), previously digested with Sall and BglII, to give the plasmid pAB083 (7436 bp) (Figure No. 15).

#### **Example 19: Construction of the Plasmid pAB041 (rabies virus G gene)**

An RT-PCR reaction according to the technique described in Example 5 was carried out with the rabies virus (ERA strain) genomic RNA (A. Anilionis et al. Nature. 1981. 294. 275-278), prepared according to the technique described in Example 3, and with the following oligonucleotides:

AB011 (33 mer) (SEQ ID No. 29)

5'AAAAGTGCAGAGATGGTTCCTCAGGCTCTCCTG3'

AB012 (34 mer) (SEQ ID No. 30)

5'CGCGGATCCTCACACTCTGGTCTCACCCCCACTC3'

so as to amplify a 1589 bp fragment containing the gene encoding the rabies virus G glycoprotein. After purification, the RT-PCR product was digested with PstI and BamHI to give a 1578 bp PstI-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and BamHI, to give the plasmid pAB041 (6437 bp) (Figure No. 16).

#### **Example 20: Production and Purification of the Plasmids**

For the preparation of the plasmids intended for the vaccination of animals, any technique may be used that allows for obtaining a suspension of purified plasmids predominantly in the supercoiled form. These techniques are well known to one skilled in the art. There may be mentioned in particular the alkaline lysis technique followed by two successive ultracentrifugations on a caesium chloride gradient in the presence of ethidium bromide as described in J. Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd edition, Cold

Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989). Reference may also be made to Patent Applications PCT WO 95/21250 and PCT WO 96/02658 which describe methods for producing, on an industrial scale, plasmids which can be used for vaccination. For the purposes of the manufacture of vaccines (see Example 17), the purified plasmids are resuspended so as to obtain solutions at a high concentration ( $>2$  mg/ml) which are compatible with storage. To do so, the plasmids are resuspended either in ultrapure water or in TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0).

#### **Example 21: Manufacture of the Associated Vaccines**

The various plasmids necessary for the manufacture of an associated vaccine are mixed using their concentrated solutions (Example 16). The mixtures are prepared such that the final concentration of each plasmid corresponds to the effective dose of each plasmid. The solutions which can be used to adjust the final concentration of the vaccine may be either a 0.9% NaCl solution, or PBS buffer.

Specific formulations such as liposomes, cationic lipids, may also be used for the manufacture of the vaccines.

#### **Example 22: Vaccination of Cats**

The cats are vaccinated with doses of 10  $\mu$ g, 50  $\mu$ g or 250  $\mu$ g per plasmid.

The injections are performed with a needle using the intramuscular route. In that case, the vaccinal doses are administered in a volume of 1 ml.

The injections can also be performed with a needle using the intradermal route. In that case, the vaccinal doses are administered in a total volume of 1 ml administered in 10 injections of 0.1 ml or at 20 injections of 0.05 ml. The intradermal administrations are performed after shaving the skin (thoracic flank in general) or at the level of a relatively glabrous anatomical region, for example the inner surface of the thigh.

A liquid jet injection apparatus (with no needle) can also be used for the intradermal injections.

## CLAIMS

1. Vaccine formula intended for cats, comprising at least three polynucleotide vaccine valencies each comprising an integrating plasmid, so as to express in vivo in the host cells, a gene of a feline pathogen valency, these valencies being selected from the group consisting of feline leukaemia virus, panleukopenia virus, infectious peritonitis virus, coryza virus, calicivirovirus, feline immunodeficiency virus and possibly rabies virus, the plasmids comprising, for each valency, one or more of the genes selected from the group consisting of env and gag for the feline leukaemia, VP2 for the panleukopenia, modified S and M for the infectious peritonitis, gB and gD for the coryza, capsid for the calicivirovirus, env and gag/pro for the feline immunodeficiency and G for the rabies.

2. Vaccine formula according to claim 1, characterized in that it comprises the panleukopenia, coryza and calicivirovirus valencies.

3. Vaccine formula according to claim 1 or 2, characterized in that it comprises the coryza virus gB and gD genes, in the same plasmid or in different plasmids, or only one of these genes.

4. Formula according to claim 1, characterized in that it comprises the feline leukaemia virus env and gag genes, in the same plasmid or indifferent plasmids, or the env gene alone.

5. Vaccine formula according to claim 1, characterized in that it comprises the two env and gag/pro genes in different plasmids or in the same plasmid, or only one of these genes.

6. Vaccine formula according to claim 1 or 2, characterized in that it comprises the M gene or the modified S gene in a plasmid or all the genes encoding M or modified S in the same plasmid or in different plasmids.

7. Vaccine formula according to any one of claims 1 to 6, characterized in that it comprises from 10 ng to 1 mg, preferably from 100 ng to 500 µg, still more preferably from 1 µg to 250 µg of each plasmid.

8. Use of one or more plasmids as described in any one of claims 1 to 7, for the manufacture of a vaccine intended to vaccinate cats primo-vaccinated by means of a first vaccine selected from the group consisting of a live whole vaccine, an inactivated whole vaccine, a subunit vaccine, a recombinant vaccine, the first vaccine having the antigen(s) encoded by the plasmid(s) or antigen(s) providing cross-protection.

9. Vaccination kit for cats assembling together a vaccine formula according to any one of claims 1 to 8 and a vaccine for cats selected from the group consisting of a live whole vaccine, an inactivated whole vaccine, a subunit vaccine, a recombinant vaccine, the first vaccine having the antigen encoded by the polynucleotide vaccine or an antigen providing cross-protection, for administration of the latter in primo-vaccination and as a booster with the vaccine formula.

1 / 19

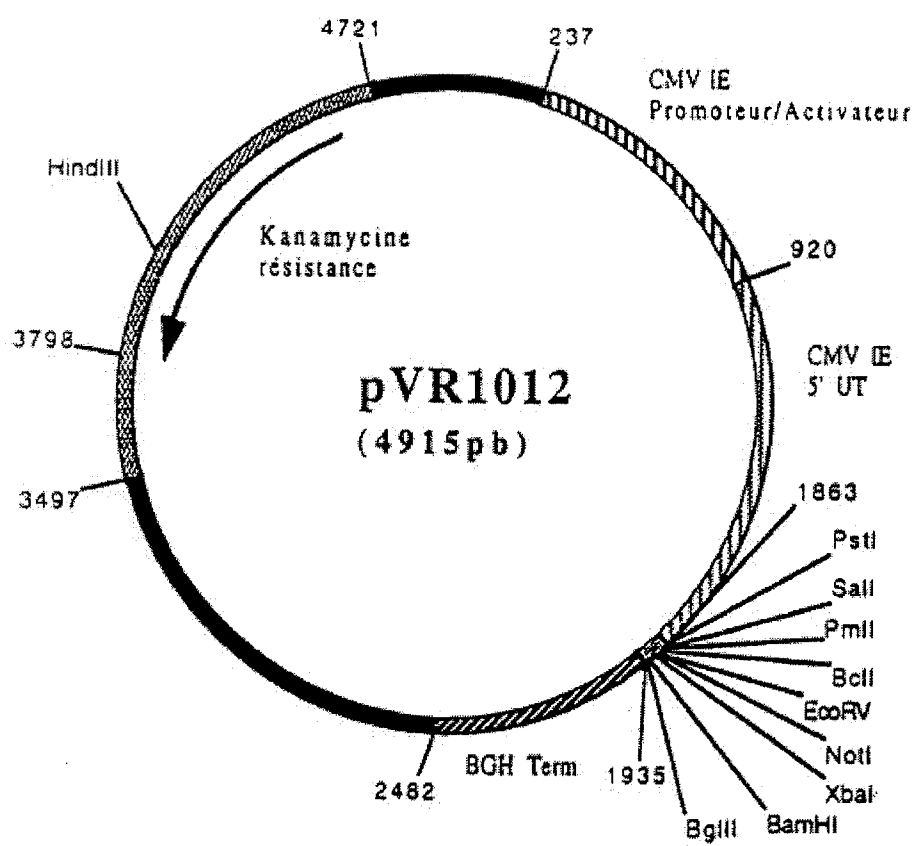


Figure No. 1

2 / 19

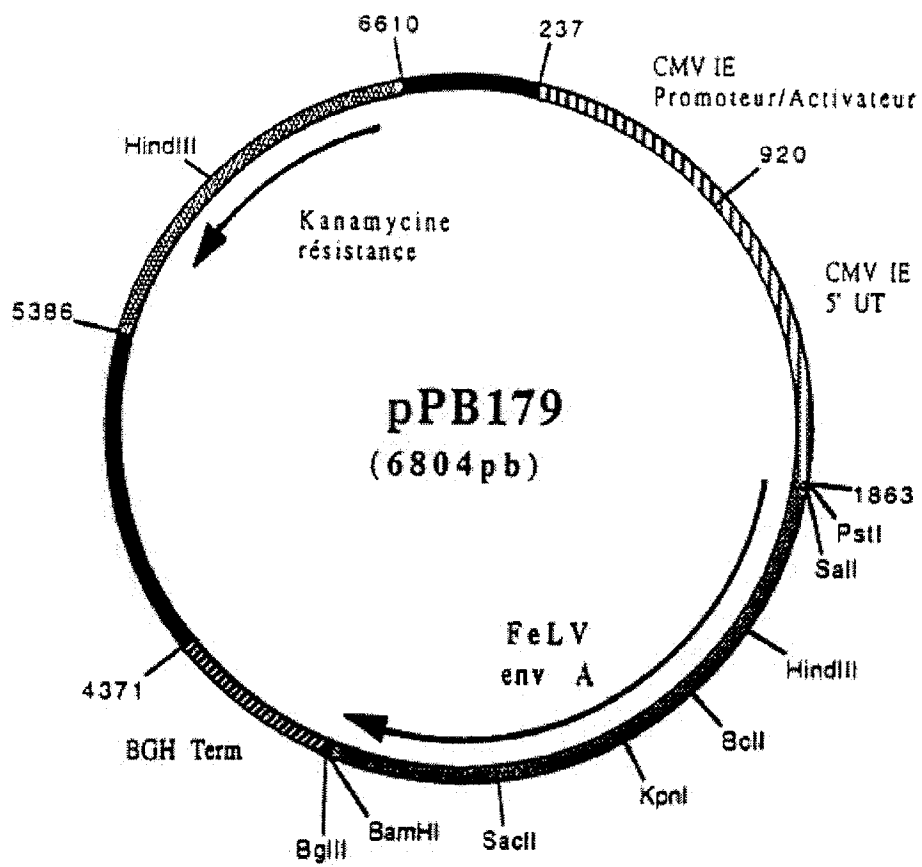


Figure No. 2

3 / 19

1 ATGGAAGGTCCAACGCACCCAAAACCTCTAAAGATAAGACTTTCTCGTGGGACCTAATGATT  
 1 MetGluGlyProThrHisProLysProSerLysAspLysThrPheSerTrpAspLeuMetIle  
 64 CTGCTGGGGGTCTTACTAAGACTGGACGTGGGAATGGCCAATCCTAGTCCGCACCAAATATAT  
 22 LeuValGlyValLeuLeuArgLeuAspValGlyMetAlaAsnProSerProHisGlnIleTyr  
 127 AATGTAACCTGGACAATAACCAACCTTGTAAGTGAACAAAGGCTAATGCCACCTCCATGTTG  
 43 AsnValThrTrpThrIleThrAsnLeuValThrGlyThrLysAlaAsnAlaThrSerMetLeu  
 190 GGAACCTGACAGACGCCTTCCCTACCATGTATTTTGACTTATGTGATATAATAGCAAATACA  
 64 GlyThrLeuThrAspAlaPheProThrMetTyrPheAspLeuCysAspIleIleGlyAsnThr  
 253 TGAACCCCTTCAGATCAAGAACCATTCCCAGGGTATGGATGTGATCAGCCTATGAGGAGCTGG  
 85 TrpAsnProSerAspGlnGluProPheProGlyTyrGlyCysAspGlnProMetArgArgTrp  
 316 CGACAGAGAAACACACCCTTTTATGTCTGTCCAGGACATGCCAACCGGAAGCAATGTGGGGGG  
 106 ArgGlnArgAsnThrProPheTyrValCysProGlyHisAlaAsnArgLysGlnCysGlyGly  
 379 CCACAGGATGGGTTTCTCGCTGTATGGGGTTCGAGACCCACCGGGGAAACCTATTGGGAGACCC  
 127 ProGlnAspGlyPheCysAlaValTrpGlyCysGluThrThrGlyGluThrTyrTrpArgPro  
 442 ACCTCCTCATGGGACTACATCAGTAAAAAAGGGGTACTCAGGGAATATATCAATGTAGT  
 148 ThrSerSerTrpAspTyrIleThrValLysLysGlyValThrGlnGlyIleTyrGlnCysSer  
 505 GGAGGTGGTTGGTGTGGGCCCTGTTACGATAAAGCTGTTCACTCCTCGACAACGGGAGCTAGT  
 169 GlyGlyGlyTrpCysGlyProCysTyrAspLysAlaValHisSerSerThrThrGlyAlaSer  
 568 GAAGGGGGCCGGTGAACCCCTTGATCTTGCAATTTACCCAAAAGGGAAGACAAACATCTTGG  
 190 GluGlyGlyArgCysAsnProLeuIleLeuGlnPheThrGlnLysGlyArgGlnThrSerTrp  
 631 GATGGACCTAAGTCATGGGGCTACGACTATACCGTTCAGGATATGACCTATAGCCCTGTTTC  
 211 AspGlyProLysSerTrpGlyLeuArgLeuTyrArgSerGlyTyrAspProIleAlaLeuPhe  
 694 TCGGTATCCCGCAAGTAATGACCATTACCGCGCTCAGGCCATGGGACCAAATCTAGTCCTG  
 232 SerValSerArgGlnValMetThrIleThrProProGlnAlaMetGlyProAsnLeuValLeu  
 757 CCTGATCAAAAACCCCATCCAGGCAATCTCAAATAGAGTCCCGAGTAACACCTCACCATTCC  
 253 ProAspGlnLysProProSerArgGlnSerGlnIleGluSerArgValThrProHisHisSer  
 820 CAAGGCAACGGAGGCACCCAGGTGTAACCTTTGTTAATGCCTCCATTGCCCCCTCTACGTACC  
 274 GlnGlyAsnGlyGlyThrProGlyValThrLeuValAsnAlaSerIleAlaProLeuArgThr  
 883 CCTGTACCCCCGCAAGTCCCAAACGTATAGGGACCGGAAATAGGTTAATAAATTTAGTGCAA  
 295 ProValThrProAlaSerProLysArgIleGlyThrGlyAsnArgLeuIleAsnLeuValGln  
 946 GGGACATACCTAGCCTTAAATGCCACCGACCCCAACAAACTAAAGACTGTTGGCTCTGCCTG  
 316 GlyThrTyrLeuAlaLeuAsnAlaThrAspProAsnLysThrLysAspCysTrpLeuCysLeu  
 1009 GTTCTCGACCACCTTATTACGAAGGGATTGCAATCTTAGGTAACACAGCAACCAACAAAC  
 337 ValSerArgProProTyrTyrGluGlyIleAlaIleLeuGlyAsnTyrSerAsnGlnThrAsn  
 1072 CCTCCCATCCTGCCTATCTACTCCGCAACATAAGCTAAGTATATCTGAGGTGTCAGGGCAA  
 358 ProSerProSerCysLeuSerThrProGlnHisLysLeuThrIleSerGluValSerGlyGln  
 1135 GGACTGTGCATAGGGACTGTTCTTAAGACCCACCAGGCTTTGTGCAATAAGACACAACAGGGA  
 379 GlyLeuCysIleGlyThrValProLysThrHisGlnAlaLeuCysAsnLysThrGlnGlnGly  
 1198 CATACAGGGGCTCACTATCTAGCCGCCCCCAATGGCACCTATTGGGCTGTAACTGGACTC  
 400 HisThrGlyAlaHisTyrLeuAlaAlaProAsnGlyThrTyrTrpAlaCysAsnThrGlyLeu

Figure No. 3



4 / 19

1261 ACCCCATGCATTTCCATGGCAGTGCTCAATTGGACCTCTGATTTTTGTGTCTTAATCGAATTA  
 421> ThrProCysIleSerMetAlaValLeuAsnTrpThrSerAspPheCysValLeuIleGluLeu  
 1324 TGGCCCAGAGTGACCTACCATCAACCCGAATACATTTACACACATTTCCGACAAAGCTGTCAGG  
 442> TrpProArgValThrTyrHisGlnProGluTyrIleTyrThrHisPheAspLysAlaValArg  
 1387 TTCCGAAGAGAACCAATATCACTAACC GTTGCCCTTATAATGGGAGGACTCACTGTAGGGGGC  
 463> PheArgArgGluProIleSerLeuThrValAlaLeuIleMetGlyGlyLeuThrValGlyGly  
 1450 ATAGCCGCGGGGGTTCGGAACAGGGACTAAAGCCCTCCTTGAAACAGCCCAGTTCAGACAACATA  
 484> IleAlaAlaGlyValGlyThrGlyThrLysAlaLeuLeuGluThrAlaGlnPheArgGlnLeu  
 1513 CAAATGGCTATGCACGCAGACATCCAGGCCCTAGAAGAGTCAATTAGTGCCTTAGAAAAATCC  
 505> GlnMetAlaMetHisAlaAspIleGlnAlaLeuGluGluSerIleSerAlaLeuGluLysSer  
 1576 CTGACCTCCCTCTCCGAGGTAGTCTTACAAAATAGACGGGCCCTAGATATTCTGTTCTTACAA  
 526> LeuThrSerLeuSerGluValValLeuGlnAsnArgArgGlyLeuAspIleLeuPheLeuGln  
 1639 AAGGGAGGGCTCTGTGCCGCCCTTAAAGGAAGAATGCTGCTTCTATGCAGATCACACCGGACTC  
 547> LysGlyGlyLeuCysAlaAlaLeuLysGluGluCysCysPheTyrAlaAspHisThrGlyLeu  
 1702 GTCAGAGACAATATGGCTAAATTAAGAGAAAGACTGAAACAGCGACAACAACACTGTTTGACTCC  
 568> ValArgAspAsnMetAlaLysLeuArgGluArgLeuLysGlnArgGlnGlnLeuPheAspSer  
 1765 CAACAGGGATGGTTTGAAGGATGGTTCAACAAGTCCCCCTGGTTTACAACCCTAATTTCTCTCC  
 589> GlnGlnGlyTrpPheGluGlyTrpPheAsnLysSerProTrpPheThrThrLeuIleSerSer  
 1828 ATTATAGGCCCCCTTACTAATCCTACTCCTAATTCTCCTCTTCGGCCCCATGCATCCTTAACCGA  
 610> IleIleGlyProLeuLeuIleLeuLeuLeuIleLeuLeuPheGlyProCysIleLeuAsnArg  
 1891 TTAGTGCAATTCGTAAAAGACAGAATATCTGTGGTACAAGCCTTAATTTTAACCCAACAGTAC  
 631> LeuValGlnPheValLysAspArgIleSerValValGlnAlaLeuIleLeuThrGlnGlnTyr  
 1954 CAACAGATACAGCAATATGATCCGGACCGACCATGA  
 652> GlnGlnIleGlnGlnTyrAspProAspArgPro...

Figure No. 3 (last part)

5 / 19

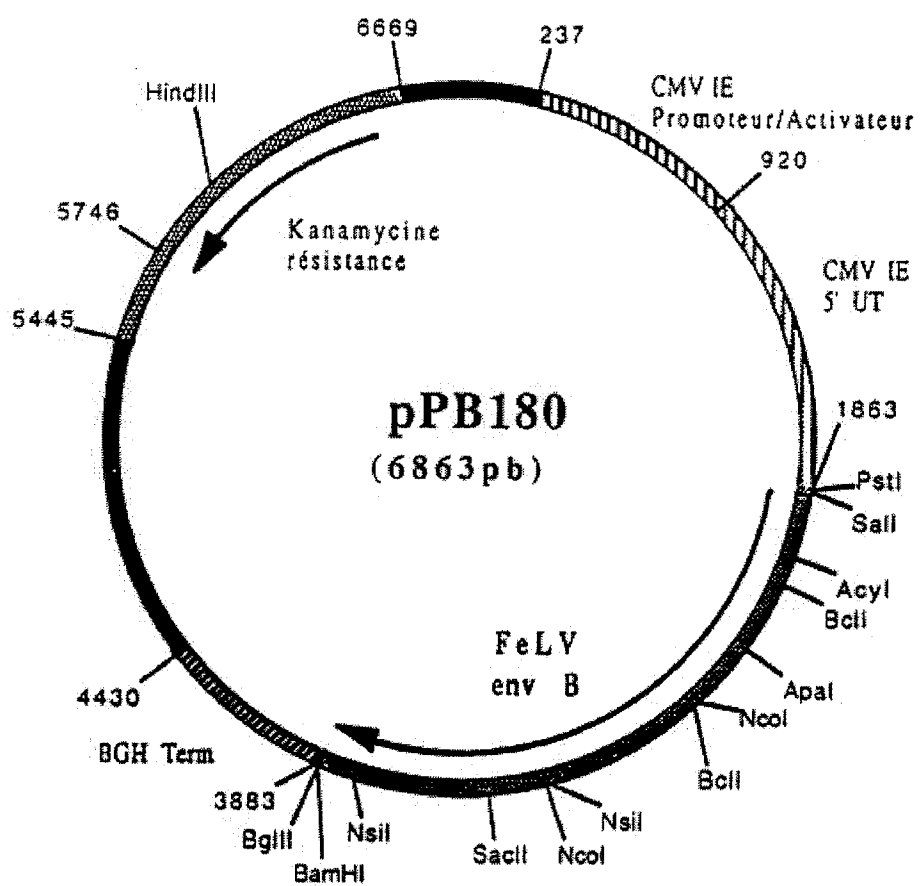


Figure No. 4

6 / 19

1 ATGTCTGGAGCCTCTAGTGGGACAGCCATTGGGGCTCATCTGTTTGGCGTCTCACCTGAATAC  
 1> MetSerGlyAlaSerSerGlyThrAlaIleGlyAlaHisLeuPheGlyValSerProGluTyr  
 64 AAGGTGTTGATCGGAGACGAGGGAGCCGGACCCCTCAAGGTCTCTTTCTGAGGTTTCATTTTTCG  
 22> ArgValLeuIleGlyAspGluGlyAlaGlyProSerArgSerLeuSerGluValSerPheSer  
 127 GTTTGGTACCAAAGACGCCGGGCACGTCTTGTCATTTTTTGTCTGGTTGCGTCTTTTCTTGTC  
 43> ValTrpTyrGlnArgArgAlaAlaArgLeuValIlePheCysLeuValAlaSerPheLeuVal  
 190 CCTTGTCTAACCTTTTTAATTGCAGAAACCGTCATGGGCCAACTATAACTACCCCTTAAGC  
 64> ProCysLeuThrPheLeuIleAlaGluThrValMetGlyGlnThrIleThrThrProLeuSer  
 253 CTCACCCTTGATCACTGGTCTGAAGTCCGGGCACGAGCCCATATCAAGGTGTCGAGGTCCGG  
 85> LeuThrLeuAspHisTrpSerGluValArgAlaArgAlaHisAsnGlnGlyValGluValArg  
 316 AAAAAGAAATGGATTACCTTATGTGAGGCCGAATGGGTGATGATGAATGTGGGCTGGCCCCGA  
 106> LysLysLysTrpIleThrLeuCysGluAlaGluTrpValMetMetAsnValGlyTrpProArg  
 379 GAAGGAACCTTTTCTCTTGATAGCATTTCCAGGTTGAAAAGAAGATCTTCGCCCGGGACCA  
 127> GluGlyThrPheSerLeuAspSerIleSerGlnValGluLysLysIlePheAlaProGlyPro  
 442 TATGGACACCCCGACCAAGTTCCTTACATTACTACATGGAGATCCTTAGCCACAGACCCCT  
 148> TyrGlyHisProAspGlnValProTyrIleThrThrTrpArgSerLeuAlaThrAspProPro  
 505 TCGTGGGTTCTGTCGGTTCCTACCCCTCCCAACCTCCACACCCCTCCCTCAACCTCTTTTCG  
 169> SerTrpValArgProPheLeuProProProLysProProThrProLeuProGlnProLeuSer  
 568 CCGCAGCCCTCCGCCCCCTCTTACCTCTTCCCTCTACCCCGTTCTCCCCAAGCCAGACCCCCC  
 190> ProGlnProSerAlaProLeuThrSerSerLeuTyrProValLeuProLysProAspProPro  
 631 AAACCGCCTGTGTTACCGCCTGATCCTTCTTCCCTTTAATTGATCTCTTAACAGAAGAGCCA  
 211> LysProProValLeuProProAspProSerSerProLeuIleAspLeuLeuThrGluGluPro  
 694 CCTCCCTATCCGGGGGGTACGGGCCACCGCCATCAGGTCCTAGGACCCCAACCGCTTCCCCG  
 232> ProProTyrProGlyGlyHisGlyProProProSerGlyProArgThrProThrAlaSerPro  
 757 ATTGCAAGCCGGCTAAGGGAACGACGAGAAAACCTGCTGAAGAATCGCAAGCCCTCCCTTG  
 253> IleAlaSerArgLeuArgGluArgArgGluAsnProAlaGluGluSerGlnAlaLeuProLeu  
 820 AGGGAAGGCCCCAACAACCGACCCAGTATTGGCCATTCTCAGCTTCAGACTTGATAACTGG  
 274> ArgGluGlyProAsnAsnArgProGlnTyrTrpProPheSerAlaSerAspLeuTyrAsnTrp  
 883 AAGTCGCATAACCCCTTTCTCCCAAGATCCAGTGGCCCTAACTAACCTAATTGAGTCCATT  
 295> LysSerHisAsnProProPheSerGlnAspProValAlaLeuThrAsnLeuIleGluSerIle  
 946 TTAGTGACGCATCAACCAACCTGGGACGACTGCCAGCAGCTCTTGACGGCACTCCTGACAGGC  
 316> LeuValThrHisGlnProThrTrpAspAspCysGlnGlnLeuLeuGlnAlaLeuLeuThrGly  
 1009 GAAGAAAGGCAAAGGGTCTTCTTGAGCCCCGAAGCAGGTTCCAGGCGAGGACGGACGGCCA  
 337> GluGluArgGlnArgValLeuLeuGluAlaArgLysGlnValProGlyGluAspGlyArgPro  
 1072 ACCCAACTACCAATGTCAATTGACGAGACTTTCCCTTGACCCGTCCCAACTGGGATTTTGCT  
 358> ThrGlnLeuProAsnValIleAspGluThrPheProLeuThrArgProAsnTrpAspPheAla  
 1115 ACGCCGGCAGGTAGGGAGCACCTACGCCCTTATCGCCAGTTGCTATTAGCGGGTCTCCGGGG  
 379> ThrProAlaGlyArgGluHisLeuArgLeuTyrArgGlnLeuLeuLeuAlaGlyLeuArgGly

Figure No. 5

7 / 19

1193 GCTCCAAGACGCCCCACTAATTTGGCACAGGTAAAGCAGGTTGTACAAGGGAAAGAGGAAACG  
 400 ▶ AlaAlaArgArgProThrAsnLeuAlaGlnValLysGlnValValGlnGlyLysGluGluThr  
 1261 CCAGCAGCATTTTTAGAAAGATTAAAAGAGGCTTATAGAATGTACACTCCCTATGACCCTGAG  
 421 ▶ ProAlaAlaPheLeuGluArgLeuLysGluAlaTyrArgMetTyrThrProTyrAspProGlu  
 1324 GACCCAGGGCAAGCGGCTAGTGTTCCTATCCTTTATATACCAGTCTAGCCAGATATAAGA  
 442 ▶ AspProGlyGlnAlaAlaSerValIleLeuSerPheIleTyrGlnSerSerProAspIleArg  
 1387 AATAAGTTACAAAGGCTAGAAGGCCTACAAGGGTTCACCCTATCTGATCTGCTAAAAGAGGCA  
 463 ▶ AsnLysLeuGlnArgLeuGluGlyLeuGlnGlyPheThrLeuSerAspLeuLeuLysGluAla  
 1450 GAAAAGATATACAACAAAAGGGAGACCCAGAGGAAAGGGAAGAAAGATTATGGCAGCGACAG  
 484 ▶ GluLysIleTyrAsnLysArgGluThrProGluGluArgGluGluArgLeuTrpGlnArgGln  
 1513 GAAGAAAGAGATAAAAAGCGCCACAAGGAGATGACTAAAGTTCTGGCCACAGTAGTTGCTCAG  
 505 ▶ GluGluArgAspLysLysArgHisLysGluMetThrLysValLeuAlaThrValValAlaGln  
 1576 AATAGAGATAAGGATAGAGAAGAAAGTAACTGGGGGATCAAAGGAAAATACCTCTGGGGAAA  
 526 ▶ AsnArgAspLysAspArgGluGluSerLysLeuGlyAspGlnArgLysIleProLeuGlyLys  
 1639 GACCAAGTGTGCCTATTGCAAGGAAAAGGGGCATTGGGGTTCGCGATTGCCCCAAACGACCCAGG  
 547 ▶ AspGlnCysAlaTyrCysLysGluLysGlyHisTrpValArgAspCysProLysArgProArg  
 1702 AAGAAACCCGCCAACTCCACTCTCCTCACTTAGGAGATTAGGAGAGTCAGGGCCAGGACCCC  
 568 ▶ LysLysProAlaAsnSerThrLeuLeuAsnLeuGlyAsp...  
 1 ▶ GluIleArgArgValArgAlaArgThrPr  
 1765 CCCCCCTGAGCCCAGGATAACCTTAAAAATAGGGGGCAACCGGTGACTTTTCTGGTGGAC  
 10 ▶ oProProGluProArgIleThrLeuLysIleGlyGlyGlnProValThrPheLeuValAspTh  
 1828 GGGAGCCCAGCACTCAGTACTGACTCGACCAGATGGACCTCTCAGTGACCGCACAGCCCTGGT  
 31 ▶ rGlyAlaGlnHisSerValLeuThrArgProAspGlyProLeuSerAspArgThrAlaLeuVa  
 1891 GCAAGGAGCCACGGGAAGCAAAAACCTACCGGTGGACCACCGACAGGAGGGTACAACCTGGCAAC  
 52 ▶ lGlnGlyAlaThrGlySerLysAsnTyrArgTrpThrThrAspArgArgValGlnLeuAlaTh  
 1954 CGGTAAGGTGACTCATTCTTTTTTATATGTACCTGAATGTCCCTACCCGTTATTAGGGAGAGA  
 73 ▶ rGlyLysValThrHisSerPheLeuTyrValProGluCysProTyrProLeuLeuGlyArgAs  
 2017 CCTATTAACTAACTTAAGGCCCAAATCCATTTTACCGGAGAAGGGGCTAATGTTGTTGGGCC  
 94 ▶ pLeuLeuThrLysLeuLysAlaGlnIleHisPheThrGlyGluGlyAlaAsnValValGlyPr  
 2080 CAGGGGTTTACCCCTACAAGTCCTTACTTTACAATTAGAAGAGGAGTATCGGCTATTTGAGCC  
 115 ▶ oArgGlyLeuProLeuGlnValLeuThrLeuGlnLeuGluGluGluTyrArgLeuPheGluPr  
 2143 AGAAAGTACACAAAAACAGGAGATGGACACTTGGCTTAAAACTTTCCCCAGGCGTGGGCAGA  
 136 ▶ oGluSerThrGlnLysGlnGluMetAspThrTrpLeuLysAsnPheProGlnAlaTrpAlaGl

Figure No. 5 (middle part)

8 / 19

2206 AACAGGAGGTATGGGAATGGCTCATTGTCAAGCCCCCTTCTCATTCAACTTAAGGCTACTGC  
 157▶ uThrGlyGlyMetGlyMetAlaHisCysGlnAlaProValLeuIleGlnLeuLysAlaThrAl  
 2269 CACTCCAATCTCCATCCGACAGTATCCTATGCCCCATGAAGCGTACCAGGGAATTAAGCCTCA  
 178▶ aThrProIleSerIleArgGlnTyrProMetProHisGluAlaTyrGlnGlyIleLysProHi  
 2332 TATAAGAAGAATGCTAGATCAAGGCATCCTCAAGCCCTGCCAGTCCCCATGGAATACACCCTT  
 199▶ aIleArgArgMetLeuAspGlnGlyIleLeuLysProCysGlnSerProTrpAsnThrProLe  
 2395 ATTACCTGTTAAGAAGCCAGGGACCGAGGATTACAGACCAGTGCAGGACTTAAGAGAAGTAAA  
 220▶ uLeuProValLysLysProGlyThrGluAspTyrArgProValGlnAspLeuArgGluValAs  
 2458 CAAAAGAGTAGAAGACATCCATCCTACTGTGCCAAATCCATATAACCTCCTTAGCACCCCTCCC  
 241▶ nLysArgValGluAspIleHisProThrValProAsnProTyrAsnLeuLeuSerThrLeuPr  
 2521 GCCGTCTCACCCCTTGGTACACTGTCCTAGATTTAAAGGACGCTTTTTTCTGCCTGCGACTACA  
 262▶ oProSerHisProTrpTyrThrValLeuAspLeuLysAspAlaPhePheCysLeuArgLeuHi  
 2584 CTCTGAGAGTCAGTTACTTTTTGCATTTGAATGGAGAGATCCAGAAATAGGACTGTCAGGGCA  
 283▶ sSerGluSerGlnLeuLeuPheAlaPheGluTrpArgAspProGluIleGlyLeuSerGlyGI  
 2647 ACTAACCTGGACACGCCTTCCTCAGGGGTTCAGAATAGCCCCACCCTATTTGATGAGGCCCT  
 304▶ nLeuThrTrpThrArgLeuProGlnGlyPheLysAsnSerProThrLeuPheAspGluAlaLe  
 2710 GCACTCAGACCTGGCCGATTTTCAGGGTAAGGTACCCGGCTCTAGTCCTCCTACAATATGTAGA  
 325▶ uHisSerAspLeuAlaAspPheArgValArgTyrProAlaLeuValLeuLeuGlnTyrValAs  
 2773 TGACCTCTTGCTGGCTGCGGCAACCAGGACTGAATGCCTGGAAGGGACTAAGGCACTCCTTGA  
 346▶ pAspLeuLeuLeuAlaAlaAlaThrArgThrGluCysLeuGluGlyThrLysAlaLeuLeuGI  
 2836 GACTTTGGGCAATAAGGGGTACCGAGCCTCTGGAAAGAAGGCCCAAATTTGCCTGCAAGAAGT  
 367▶ uThrLeuGlyAsnLysGlyTyrArgAlaSerGlyLysLysAlaGlnIleCysLeuGlnGluVa  
 2899 CACATACCTGGGGTACTCTTTAAAAGATGCCCAAAGGTGGCTTACCAAAGCTCGGAAAGAAGC  
 388▶ lThrTyrLeuGlyTyrSerLeuLysAspGlyGlnArgTrpLeuThrLysAlaArgLysGluAl  
 2962 CATCCTATCCATCCCTGTGCCTAAAAACCCACGACAACCTGAGAGAGTTCCTTGGAACTGCAG  
 409▶ aIleLeuSerIleProValProLysAsnProArgGlnValArgGluPheLeuGlyThrAla

Figure No. 5 (Last part)

9 / 19

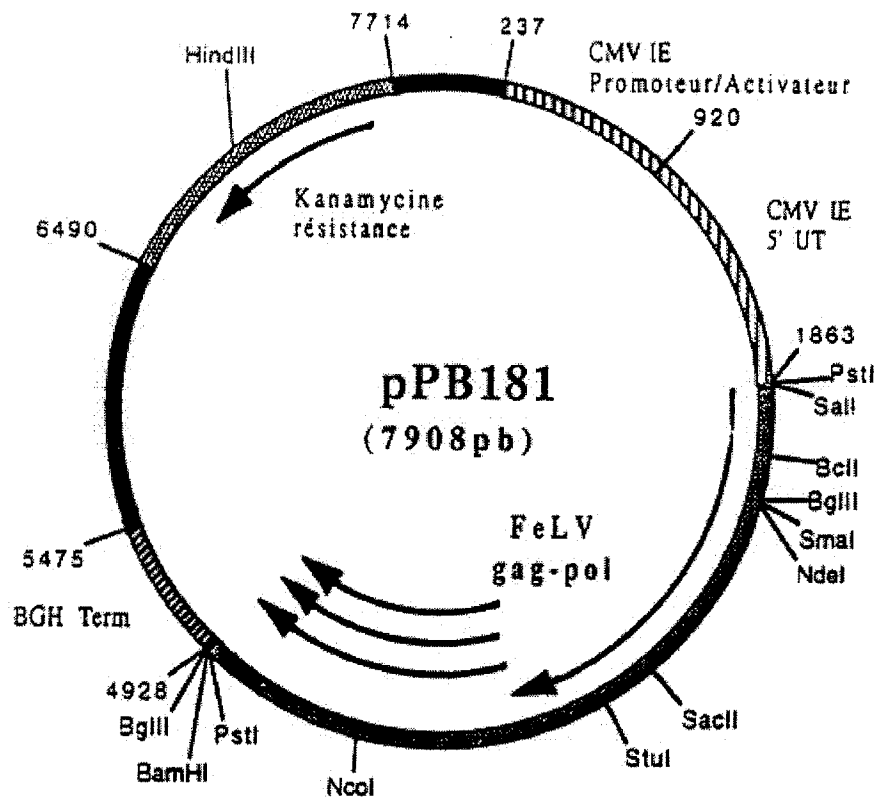


Figure No. 6

10 / 19

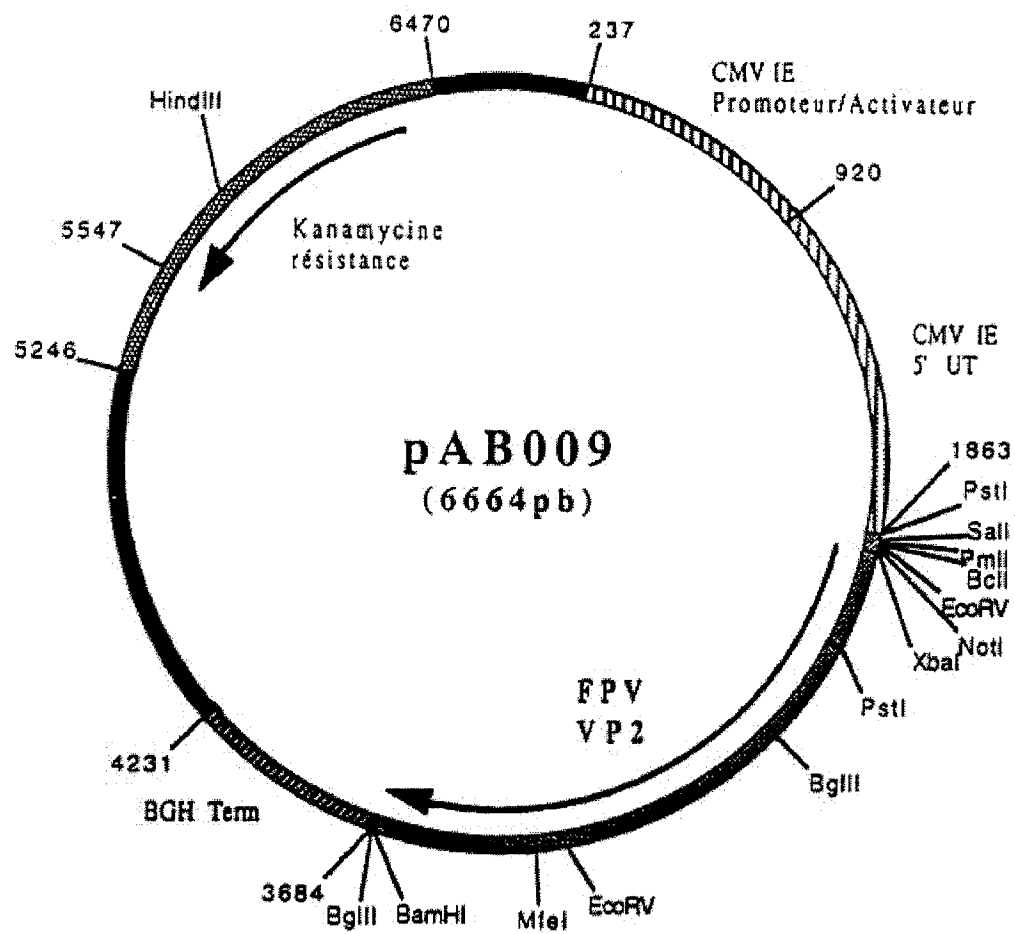


Figure No. 7

11 / 19

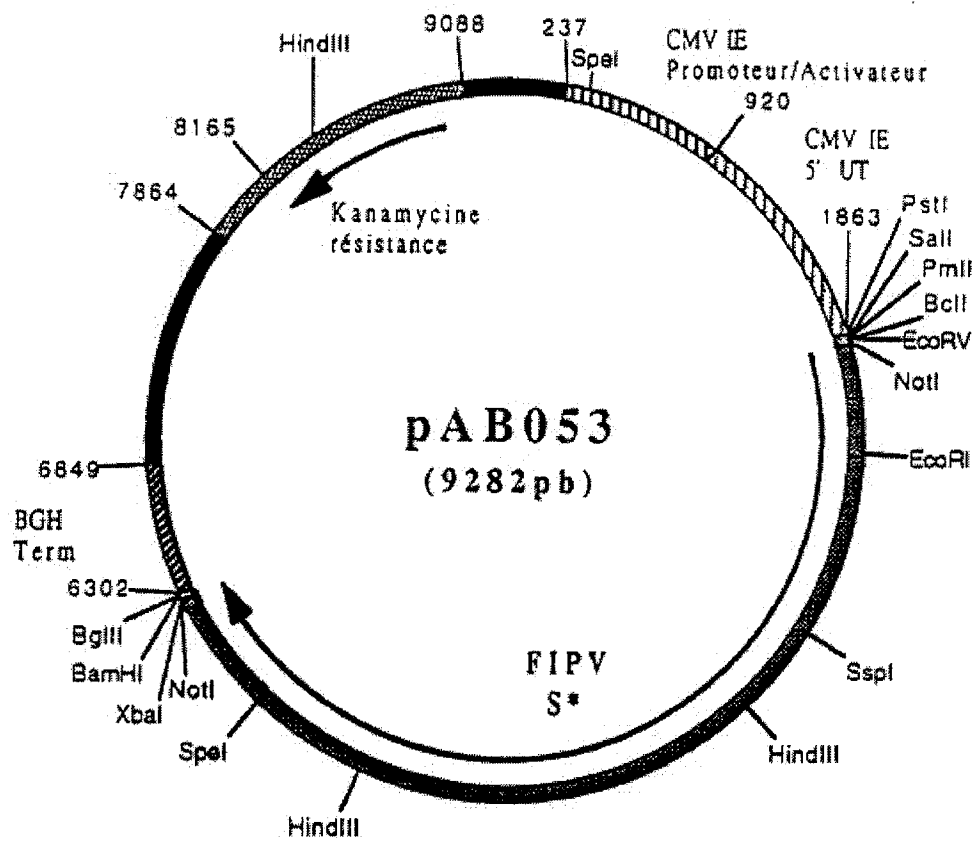


Figure No. 8



12 / 19

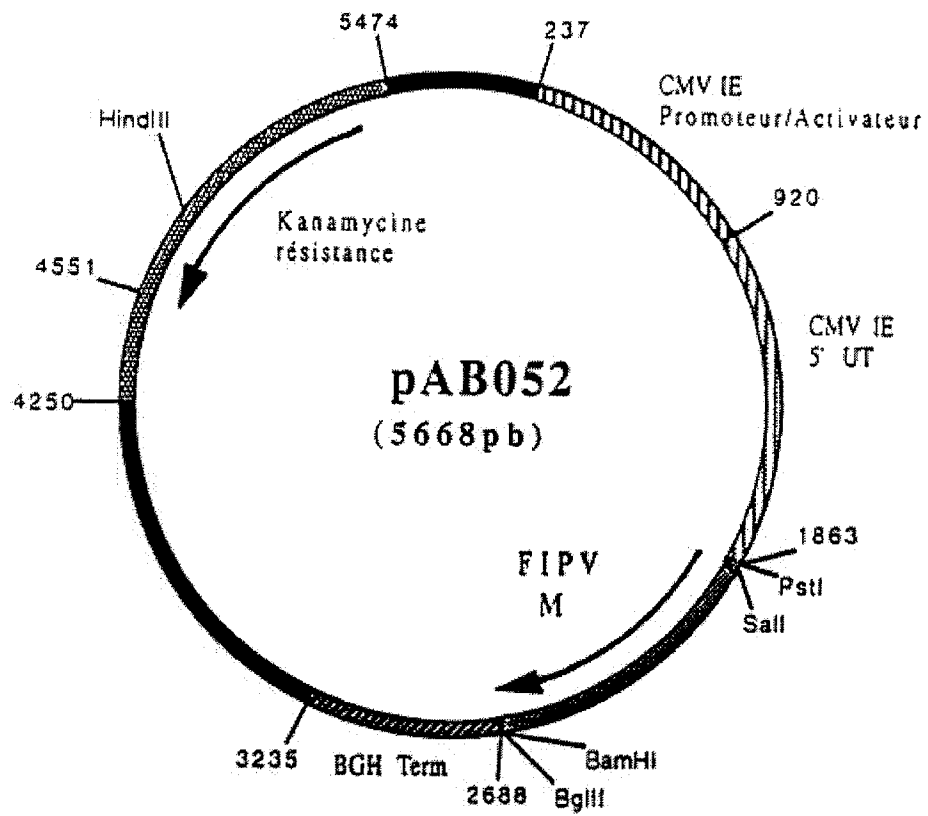


Figure No. 9

13 / 19

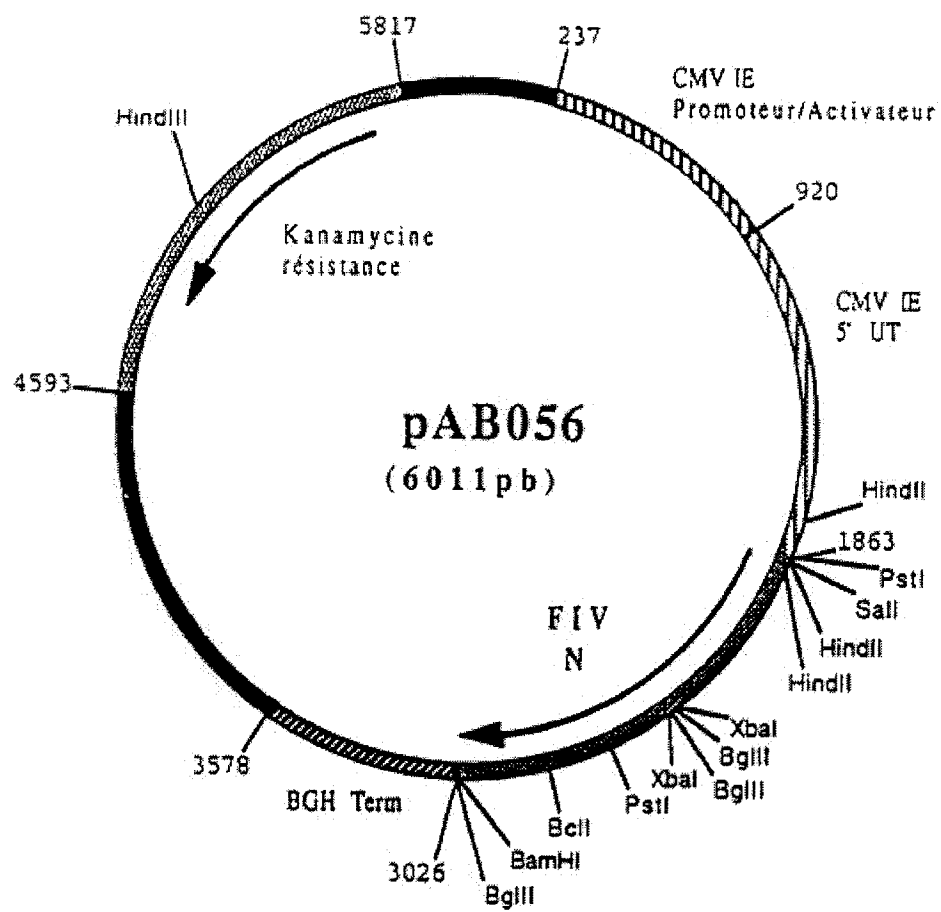


Figure No. 10

14 / 19

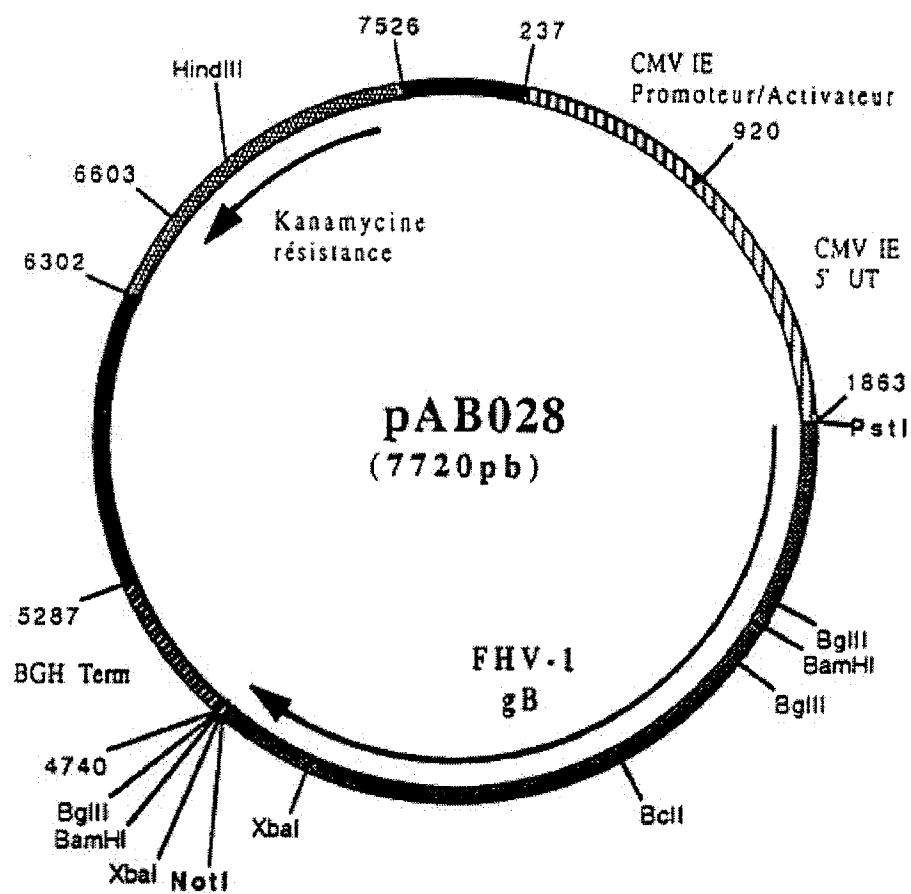


Figure No. 11

15 / 19

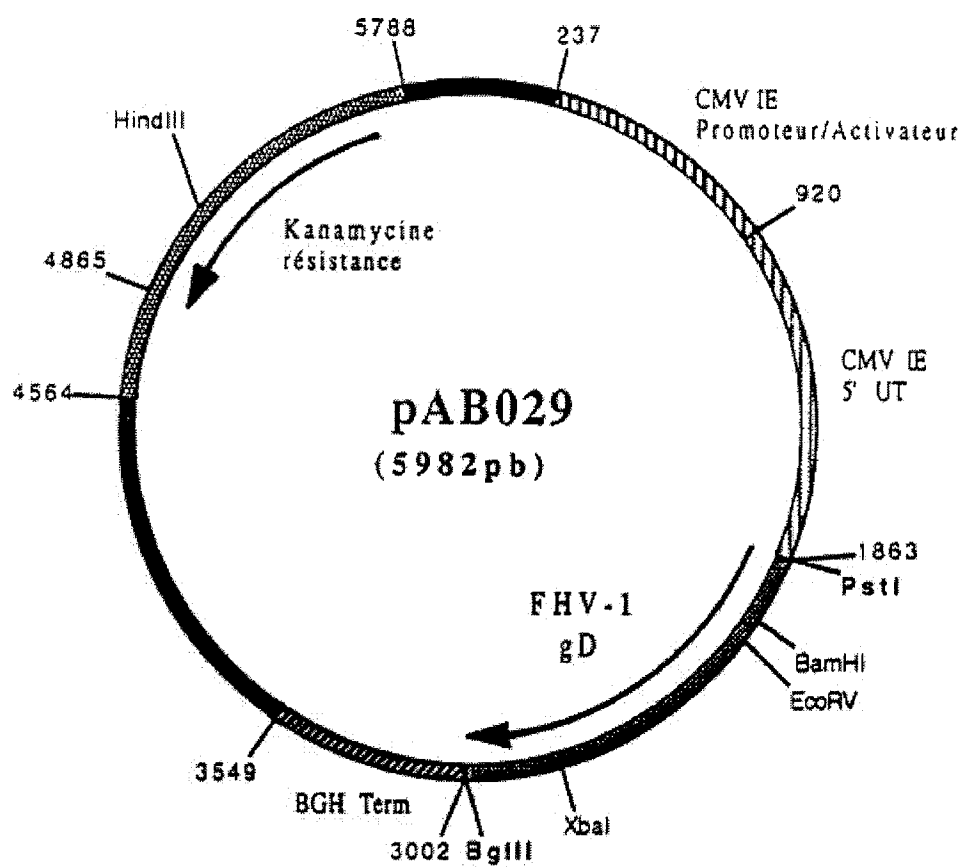


Figure No. 12

16 / 19

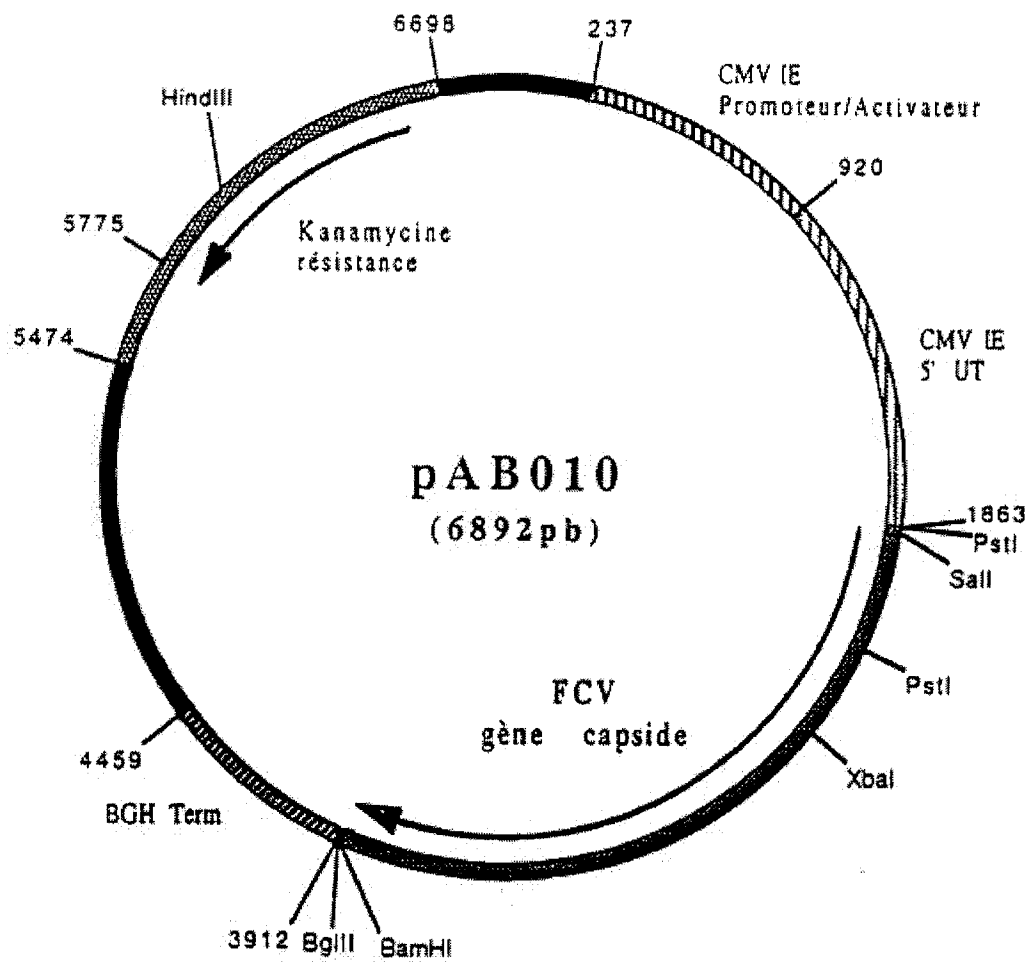


Figure No. 13

17 / 19

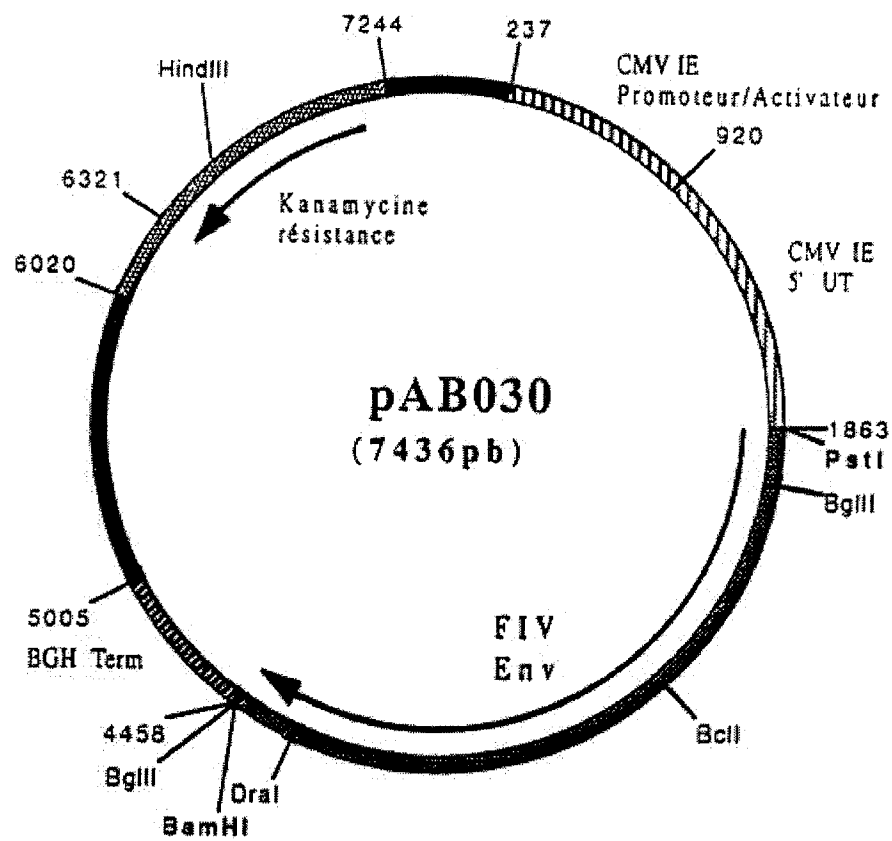


Figure No. 14

18 / 19

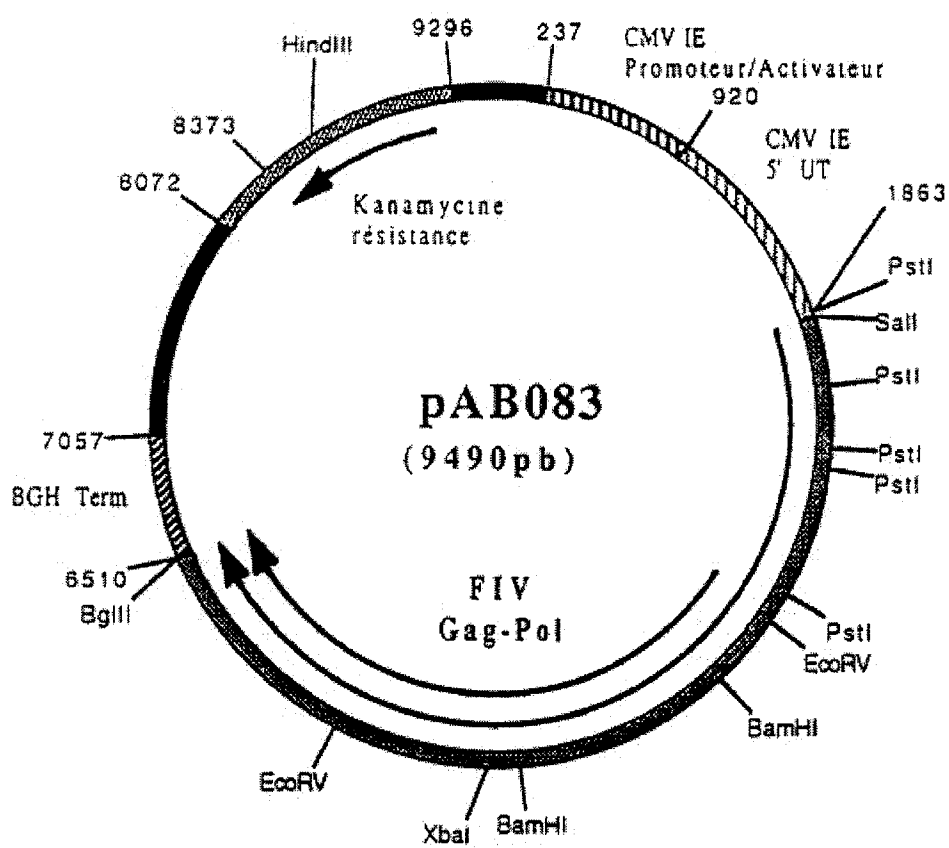


Figure No. 15

19 / 19

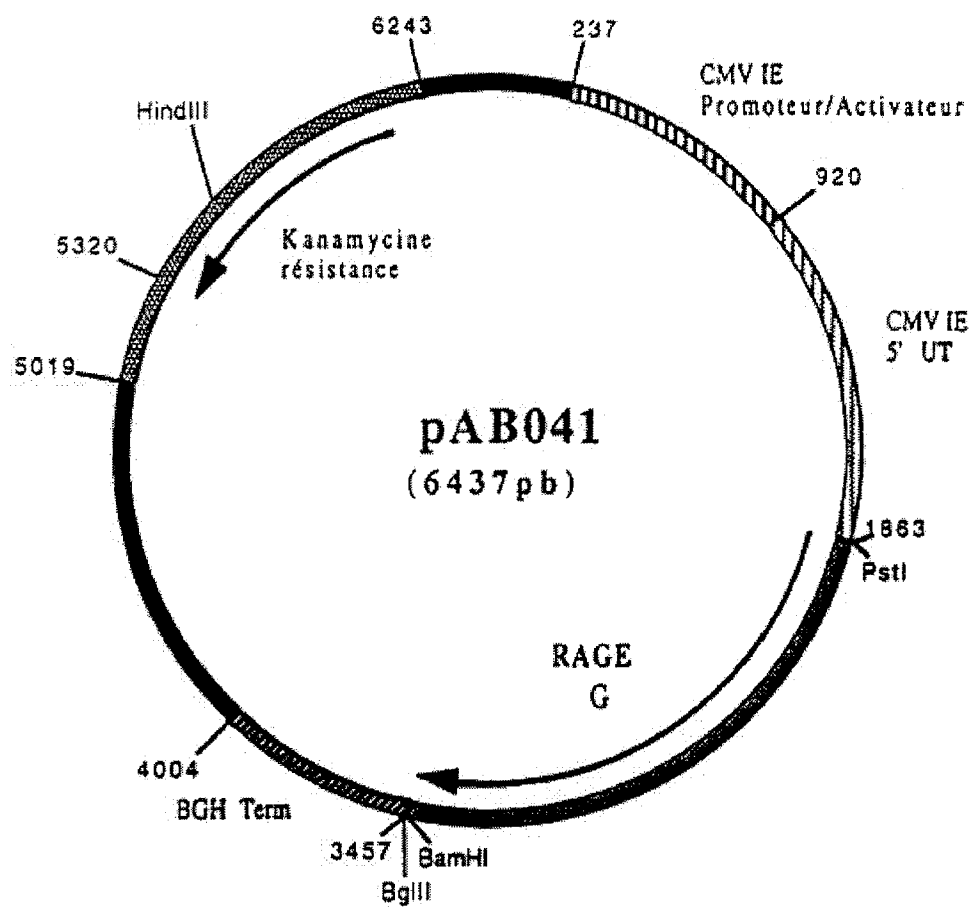


Figure No. 16